AN INVITRO EVALUATION OF REMINERALIZING EFFECT OF NANOHYDROXYAPATITE ON EARLY ENAMEL LESIONS - AN AFM STUDY

Dissertation submitted to

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In partial fulfillment for the Degree of

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CERTIFICATE

This is to certify that this dissertation titled **AN INVITRO EVALUATION OF REMINERALIZING EFFECT OF NANOHYDROXYAPATITE ON EARLY ENAMEL LESIONS - AN AFM STUDY** is a bonafide record work done by **Dr. ANN JOHNS** under our guidance during her postgraduate study period between 2010 - 2013.

This dissertation is submitted to **THE TAMILNADU Dr. M.G.R. MEDICAL UNIVERSITY**, in partial fulfillment for the degree of **MASTER OF DENTAL SURGERY – CONSERVATIVE DENTISTRY AND ENDODONTICS, BRANCH IV**. It has not been submitted (partial or full) for the award of any other degree or diploma.

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LIST OF ABBREVIATIONS

NHA	Nanohydroxyapatite
SMHR	Surface mircohardness recovery
SMH	Surface microhardness
Rrms	Roughness value representing the height distribution relative to the mean line
Rz	Roughness value representing average maximum peak -to-valley height of five consecutive sampling depths
AFM	Atomic force microscope

ABSTRACT

Background:

The early enamel lesions are reversible as it is a process involving mineral transactions between the teeth and saliva.

Aim:

This study was aimed to evaluate the effect of different concentrations of nano-hydroxyapatite particles in the management of artificially created early enamel lesions.

Materials and Methods:

Artificial carious lesions were prepared in human enamel with demineralizing solution. The treatment agents included were sodium fluoride (Positive control), distilled water (Negative control), 5%wt, 8%wt and 10%wt of nano-hydroxyapatite. All the treatment agents were subjected to treatment solutions as per the pH cycling model for 12 days to simulate the daily oral environment's acid challenge. The remineralization parameters-surface hardness and surface roughness of enamel blocks were evaluated with Vickers indenter and atomic force microscope respectively.

Results:

Statistical analysis showed significant decrease in surface hardness and increase in surface roughness after subjecting the enamel blocks to nano-hydroxyapatite.

Conclusion:

Nano-hydroxyapatite can be regarded as a potential remineralising agent with the optimum concentration being 10%wt. It can be concluded as a noninvasive means of managing early enamel carious lesions.

Keywords: Early enamel lesions, Nano-hydroxyapatite, sodium fluoride, Remineralization.

INTRODUCTION

Dental caries is a chronic, worldwide oral disease that progresses slowly in most individuals. It was only in the fourteenth century that a sharp increase in the prevalence of caries was noted. Keene et al ascribed it to a sucrose-civilization-caries trinity.[8] In the past, the understanding of the pathological process behind caries was limited. Caries has been defined as a multifaceted pH mediated infectious disease initiated by a biofilm which changes dynamically with it's environment such as tooth, pellicle and saliva.[8,22] The term ***biofilm*** refers to a 3-D accumulation of interacting microorganisms on a surface embedded in an extra cellular polymer matrix.

Under normal conditions, saliva is supersaturated with calcium and phosphate ions in comparison to the mineral content of the tooth. It is the ***statherin,*** a proline rich peptide in saliva which is responsible for the stabilisation of calcium and phosphate ions on the tooth.[52]As the saliva surrounding the tooth becomes more acidic, a point is reached when it ceases to be supersaturated and any further decrease in pH results in mineral dissolution. This pH has been termed as

"Critical pH"& falls in the range of **pH 5.2-5.5** depending on the particular saliva composition of the individual.[6]

The natural defence mechanism of saliva is disturbed, when oral microorganisms form a biofilm on the enamel surface followed by exposure to fermentable dietary carbohydrates. When the pH becomes less than 5.5, loss of calcium and phosphate ions results from the hydroxyapatite layer of tooth surface. This has been termed as demineralization.[53]

Early enamel lesions with an intact surface and a porous subsurface results at a pH of 5.Dr.Robert Stephen had graphically represented the rapid drop in pH that could cause enamel demineralization. According to stephen's curve, it takes 30-60 minutes to revert back to the normal pH (6.3-7.0).[14] Remineralization can occur at this stage due to the buffering capacity of saliva resulting in precipitation of calcium and phosphate ions on to the tooth surface.[36]

The caries in enamel is unique as enamel is both acellular and avascular.[51] Enamel surface at eruption time is porous and has large amounts of carbonate, water and magnesium in it's crystal

composition. The chemical composition and structure of surface enamel becomes more amorphous with exacerbations and remissions of demineralization and remineralization. Under cariogenic conditions the natural resistance of the tooth surface provided by post-eruptive enamel maturation may not be enough to prevent the formation of a caries lesion. When demineralization of the tooth surface exceeds the mineral transactions between the crystals at the tooth surface and the surrounding environment, caries lesion starts forming.[9]

In the early enamel caries, demineralization results below the surface layer of the enamel which appears as a white–spot lesion.[48] White-spot lesion can be defined as a non-cavitated carious lesion that has reached the stage where the net subsurface mineral loss has produced changes in the optical properties of enamel, resulting in a white appearance of the enamel surface.[8]

Remineralization of the lesion surface can occur first, leading to the blockage of the communication channels that allowed the demineralization of the subsurface of the lesion. Development of a mineralized surface that is highly resistant than the sound enamel can result in a sealed, partially demineralized subsurface

lesion.[9] Concerning the formation of incipient enamel lesion, given favourable change in conditions, remineralization is possible as it is a reversible phenomenon.[29]

The traditional way of managing dental caries was by a surgical approach of drill and fill[35].Recently due to the better understanding of the mechanism of caries, a new strategy has been evolved where early enamel lesions can be addressed through a medical model of treatment rather than the traditional surgical or restorative treatment.[41]

In contrast to traditional therapies, strategies that focus on shifting the balance towards mineral uptake in the tissue will results in the repair of the lesion and contribute to the prevention of new lesion formation.[31] For more than six decades, the widespread use of fluoride has caused a decline in dental caries.[48] The prevalence of dental fluorosis and systemic complications due to chronic low level exposure to fluoride has to be taken into consideration despite it's merits.

The key to enlightened caries management is to overcome rapid decreases in pH in the dental biofilm and maintain a stable

environment that can facilitate microbial homeostasis[41]. A variety of newer remineralizing agents available in the market include agents containing fluoride, casein phospho peptide-amorphous calcium phosphate, and bioavailable calcium phosphate.[43]

The nano-hydroxyapatite in the range of 20-40 nm, which is closely approximating the features of biological apatite, can be a promising remineralizing agent.[23] In the recent years an increasing number of reports have shown the ability of nano-hydroxyapatite to remineralise artificial carious lesions after it's addition to tooth pastes, mouthwashes etc. A 4wt% nano-hydroxyapatite liquid suspension has shown excellent potential to remineralise incipient caries lesions as reported by Wang et al, where as no significant difference were found between sodium fluoride and 10 wt% of nano-hydroxyapatite according to Gao et al.[13]

Till date, there has been no studies comparing the various concentrations of 5wt%, 8wt% & 10wt% of nano-hydroxyapatite for remineralization procedures.

AIM:

The aim of this study was to evaluate the effect of different concentrations of nano-hydroxyapatite particles in the non invasive management of artificially created early enamel carious lesions.

OBJECTIVE:

The objective of the present study were

1. To evaluate the surface hardness of artificially created enamel lesions before and after remineralization using Vickers micro indenter.
2. To evaluate the effect of nano-hydroxyapatite on the surface roughness of remineralized early enamel lesions using atomic force microscope.
3. To compare the surface hardness and surface roughness effect of nano-hydroxyapatite with that of sodium fluoride on the remineralized early enamel lesions.

REVIEW OF LITERATURE

TenCate et al (1981)[39] subjected artificial lesions in bovine enamel to fluorides and di-phosphonates to investigate the mechanism of remineralization of artificial lesions. A crystalline material was deposited, having rod like morphology with a diameter of 200nm.This was evident with scanning electron microscopy experiments during remineralization. They concluded that the deposited material is mostly fluoridated hydroxyl-apatite. A two fold increase in rate of remineralization occurred after the addition of 1 ppm. Fluoride to the remineralizing solution. This was comparable with older literature on the remineralization of acid-etched enamel.

Pearce et al (1985)[30] treated overlying plaque with a mineral-enriching solution to evaluate remineralization of softened bovine enamel. In this study recovery of enamel beneath this mineral-enriched plaque was 37% of the hardness lost from pre-softening, whereas control enamel under untreated plaque amounted only 14%. They concluded that the presence of a mineral phase in the overlying plaque accounted for the in vivo enamel remineralization. During

normal plaque pH cycles, this mineral would have promoted remineralization by providing mildly supersaturated conditions.

White et al (1987)[46] examined the in-vitro effects of fluoride dentifrices on the saliva-mediated repair of early carious lesions using a pH-cycling model. The characterisation of fluoride reactivity was done by fluoride uptake, surface microhardness and lesion mineral content analyses. Among the fluoride dentifrices, the most effective in enhancing the remineralization and acid resistance of early carious lesions were the sodium and amine fluoride preparations. An increase in surface hardening of specimens was positively associated with the remineralization of the early carious lesions.

Tencate et al (1991)[38] reviewed that the rate of the naturally occurring dissolution and reprecipitation processes at the tooth saliva interface depends on the fluoride present in the oral fluids. Demineralization of enamel is inhibited by the concentrations of fluoride in the sub-ppm range. Similarly, trace amounts of fluoride can accelerate the remineralization of incipient caries lesions. The dental caries process comprises of these two mechanisms with the physiological balance between hard tissue breakdown and repair. The repair process is favourably shifted by fluoride. When fluoride is

supplied at low concentrations, fluorapatite or a fluoridated hydroxyapatite may form. Thus the driving force for both the phenomena can be termed thermodynamic.

. ***Kasas et al (1997)***[21] reviewed the application domains of the atomic force microscope. They discussed that the future of atomic force microscopy in biology is through the study of different samples, starting with the imaging of single molecules to large specimens, and ending with the imaging of tissues. For the non-biologists to appreciate the significance of these studies, medical implications should be aware of. Therefore special attention has been paid in the sample description.

Farina et al (1999)[7] compared the pattern of particle distribution in the outermost layer of the tooth surfaces using atomic force microscopy. It was observed that atomic force microscopy gives high-contrast, high-resolution images and is a key tool as a source of complementary and/or new structural information.

Wang et al (2000)[44] formulated a unique technique by which acicular nano-hydroxyapatite was used to make a new bio-mimetic composite with polyamide (polyhexamethylene-adipamide).When the

physical and chemical characteristics of the composites were tested, it was seen that these synthesized nano-hydroxyapatite crystals were similar to bone apatite in size, phase composition and crystal structure. The content of biomimetic nano-hydroxyapatite crystals can reach 65%, close to that in natural bone. In addition to this these crystals were uniformly distributed in the polymer matrix.

Jandt et al (2001)[17]evaluated the use of atomic force microscopy. He stated that more than being a tool for measuring surface topography of biomaterial surfaces, it deals with the structure, properties, dynamics and manipulation of biomaterial surfaces and interfaces. Micro and nanostructure and properties of biomaterial surfaces, molecular level interactions at biomaterial-biomolecule interfaces, interfaces between biomaterials and mineralized tissues are the other applications of atomic force microscopy.

Brambilla Eugenio et al (2001)[1] discussed on the existing fluoride compounds and their clinical applications. Sodium fluoride was mainly used for systemic fluoridation by means of water, salt, milk fluoridation and the use of fluoride supplements. For topical fluoridation, solutions, gels, toothpastes and rinses of sodium fluoride, stannous fluoride, amine fluorides, acidulated phosphate

fluoride and monofluoro-phosphate were used. More recently, difluorosilane agents in a polyurethane matrix and non-aqueous fluoride varnishes in an alcoholic solution of natural resins have come to the market. Though all of these fluoridation methods have a caries preventive action, these benefits and the ease of application are different. As fluoride is a key component of oral health promotion. A coordinated approach on a community and individual basis is needed to increase the cost-benefit ratio of prevention as fluoride is a main factor of oral health promotion.

Rosin et al (2001)[33] evaluated the role of fluoride and its mechanism of action in caries prevention. In the past, caries inhibition by fluoride was ascribed to reduced solubility due to fluoride incorporation into the enamel minerals. The literature is of the view that the provision of dissolved fluoride is the key for a successful therapy. Either fluorapatite or calcium fluoride (CaF2) (like) precipitates could be the source of this fluoride. These precipitates are formed on the enamel and in the plaque after topical fluoride application. As the dissolution of the fluoride from calcium fluoride is pH dependent, these crystalline precipitates, can act as an efficient source of free fluoride ions during the acidogenic challenge. At a later

stage, these are incorporated into the enamel as hydroxyl fluorapatite or fluorapatite.

Nuca et al (2002)[29] investigated the in-vitro and in situ remineralization of the artificial white spot lesions. Results showed that the remineralization of these artificial lesions in saturated solutions of calcium-phosphate as the artificial and natural saliva is a common finding. They also found that remineralization was enhanced by the presence of fluoride ions, especially at a low pH. When compared to in-vitro remineralization, insitu remineralization is significantly more effective.

Maria et al (2003)[24] evaluated the micro hardness and chemical composition of human tooth both in enamel and dentin. They measured at variable sites using a Vicker's diamond indenter. In this study, values for microhardness are almost same all along the enamel and dentin. The uniformity of geometrical well-shaped indentation was checked with both light and scanning electron microscopes. The characteristic X-ray energy dispersive spectroscopy was used to analyse the chemical composition of the tooth. Microhardness measurements ranged from 270 to 360 VHN for enamel and 50 to 60 VHN for dentin. Cervical zone in transverse

sections showed the highest value whereas longitudinal section showed the lowest value. The results pointed out that the difference between enamel and dentin hardness depends on the percentage of organic and inorganic materials in enamel and dentin rather than the content of Na, Cl and Mg.

JieWie et al (2003)[18] developed a combination of nanocomposite of nano-hydroxyapatite and polyamide for evaluating and investigating the bioactivity for load bearing bone-repair or substitution. The results indicated that the nano-hydroxyapatite content in the composite can reach 65 wt. % which is almost similar to the apatite content in natural bone.

Vandiver et al (2005)[42] used chemically and spatially specific high-resolution force spectroscopy to detect nano scale variation in surface charge of synthetic hydroxyapatite. They concluded that the nano scale surface properties regarding the positional measurement holds great promise in extracting the molecular origins of physicochemical processes occurring at the biomaterial interface.

Cury et al (2005)[5] in their crossover study evaluated the effect of calcium carbonate based monofluorophosphates dentifrice on

enamel remineralization. The calcium carbonate based monofluoro-phosphates dentifrice was more effective than the negative control on the enhancement of enamel remineralization, either in the presence or absence of test plaque. In this study, the silica-based dentifrice also showed greater remineralization but only in the presence of test plaque.

Zhang et al (2007)[50]coprecipitated nano-hydroxyapatite & polyamide-66 in ethanol. They evaluated the effect of nano-hydroxyapatite and polyamide-66 in composites. The linkage of nano-hydroxyapatite & polyamide-66 by hydrogen bonding was showed by Infrared spectrum result. The x-ray diffraction results revealed that the introduction of nano-hydroxyapatite to polymeric matrix weakened the crystal structure. The finding that hydrogen bonding enhanced nucleation spots was showed by a different scanning calorimetric pattern. This study also proved that the inclusion of polyamide-66 in the composite decreased with the increase of the content of nano-hydroxyapatite.

Neis et al (2007)[27] suggested the manufacturing of nano-hydroxyapatite to bioactivate bone implant surfaces. Biomimetic nano-hydroxyapatite coatings can be used to render conventional

implant materials bioactive. Along with these coatings, in-situ mineralising surfaces induced by incorporation of mineralisation seeds can also be used. They are quite promising for the better performance of the implants while complying on the economic constraints on health care.

Yamazaki et al (2007)[47]determined the effect of fluoride on 1) the demineralization of sound human enamel and 2) the progression of artificial caries-like lesions, under relevant oral conditions in an in-vitro study. In-vitro results showed that fluoride concentrations of 0.19 ppm and greater can prevent the demineralization of sound enamel. They concluded that the observed effect of fluoride on enamel demineralization will not only depend on the function of fluoride solution properties, but also depends on the caries-status of the surface of enamel. The presentation of a mechanistic model in this study proved that, in comparison to sound enamel surfaces, greater concentrations of fluoride are needed to prevent the progression of artificial caries-like lesions under invivo-like conditions. This was because the diffusion of mineral ions that facilitate remineralization is rate-limiting.

Nobre-dos-Santos et al (2007)[28] evaluated insitu effect of a dentrifice with low fluoride concentration and low pH on enamel remineralization and fluoride uptake. Enamel remineralization was assessed by determining cross-sectional microhardness. The formation of loosely as well as firmly bound fluoride was analysed on the enamel surface. The values of microhardness showed that the fluoridated dentrifices were more effective than the non-fluoridated dentrifices in remineralizing dental enamel. Another finding was that, fluoridated dentrifices were also effective in forming loosely and firmly bound fluoride on enamel.

Roveri et al (2008)[34] ***i***nvestigated on a new biomimetic carbonate hydroxyapatite nano crystals. These biomimetic nano crystals were designed and synthesized to obtain a remineralization of altered human enamel surfaces. A new deposition of carbonate-hydroxyapatite in to the eroded enamel surface was observed. They concluded this as the remineralization effect induced by biomimetic carbonate hydroxyapatite nanocrystals. This new deposition formed a persistent biomimetic mineral coating which covered and protected the enamel structure.

Li et al (2008)[23] investigated on the enamel repair using hydroxyapatite nanoparticles as the building blocks. The enamel repair were subjected to scanning electron microscopy, confocal laser scanning microscopy, quantitative measurement of the adsorption, dissolution kinetics, and nano indentation. The results showed that by using 20 nm particles as the repairing agent, there was strong affinity, excellent biocompatibility, mechanical improvement, and the enhancement of erosion-free surface. when conventional hydroxylapatite and amorphous calcium phosphate are applied, these excellent in-vitro repair effects cannot be appreciated. They concluded that nano-hydroxyapatite particle with a size of 20 nm has similar properties to the natural building blocks of enamel. Thus it may be used as an excellent repair material and anticaries agent.

Cury Jaime Aparecidoet al (2008)[4]evaluated the effect of low fluoride dentifrice on enamel de- and remineralization. Surface and cross-sectional microhardness were used to assess the enamel mineral loss or gain. Polarized light microscopy was used to analyse the lesion depth. The pH-cycling models revealed fluoride dose-response effect either by reducing enamel demineralization or enhancing remineralization. This study also evaluated the efficacy of the low F

dentifrice which presented anti-caries potential. It was not equivalent to the dentifrices containing 1,100 μg F/g.

Tencate et al (2008)[36] investigated on remineralization of deep enamel dentine caries lesions. They addressed the hypothesis whether deep lesions extending into dentine, can be remineralized under optimal conditions. They also assessed whether the process is influenced by agents affecting calcium phosphate precipitation and dissolution. The deposition in the outer enamel was evident with fluoride and bisphosphonate treatments. Previously, it was assumed that this can affect the diffusion of ions to inner layers. But it was observed that the treatments had no influence on remineralization in the deeper enamel or dentinal parts of the lesions.

Nakashima syozi et al (2009)[26] investigated on the efficacy of a test dentrifice containing nano sized Calcium carbonate on enamel lesion. The experimental dentifrice showed potential to remineralize early enamel lesions. This was due to the unique properties of nano sized calcium carbonate, which got retained on the oral surfaces and released calcium ions in to the oral fluids.

Huang et al (2009)[13] did an in-vitro analysis on the effect of nano-hydroxyapatite concentration on remineralization of initial enamel lesions. Early enamel lesions were prepared in bovine enamel with an acidic buffer. The selected treatment agents were sodium fluoride (positive control), deionized water (negative control) and four different concentrations of nano-hydroxyapatite (1%, 5%, 10% and 15% wt. %). Surface microhardness and % surface microhardness recovery increased with increasing nano-hydroxyapatite concentrations. The nano-hydroxyapatite particles were evenly deposited on the cellular structure of the demineralized enamel surface in the scanning electron microscopic analysis. These particles were observed to form new surface layers. They concluded that a 10% nano-hydroxyapatite concentration can be regarded as optimal for remineralization of early enamel caries.

Chuenarrom et al (2009)[3] determined the effect of differences in the indentation load and time on the Knoop and Vickers hardness numbers (KHN and VHN) for enamel and dentin. Twenty molar teeth were divided into twenty enamel and twenty dentin specimens. At different loads and times, each specimen was tested using a Knoop or Vickers microhardness tester. The results indicated that a difference

of indentation time was not influencing the microhardness number of enamel and dentin. The knoop hardness number values of enamel and the Vickers hardness number values of dentin were affected by variation of test loads.

***Zhang et al (2009)*[49]** investigated the effect of Galla chinensis on the surface topography of early enamel carious lesion by using atomic force microscope (AFM). Three dimensional atomic force microscopic images revealed the surface topographical changes of Gallachinensis treated enamel. Significant difference was observed among the groups before and after the pH-cycling. The atomic force microscope proved to be a powerful tool for enamel de-/remineralization research. The surface roughness results pointed out the evidences to remineralization of carious lesion. The results also showed the potential of Gallachinensis in enhancing the remineralization.

***Walsh et al (2009)*[43]** in his review on contemporary technologies on remineralization therapies, have discussed about various technologies that have value for remineralization of enamel and dentine. Ideal requisites of a remineralizing material are diffusion in to the subsurface without delivering an excess of calcium. The

authors emphasises that remineralization as a natural repair process for non cavitated lesions. It depends on calcium and phosphate ions along with fluoride, to reconstruct a new surface on available crystal remnants in subsurface lesions after demineralization.

***Hornby et al (2009)*[12]** evaluated the benefits of a new toothpaste containing hydroxyapatite and sodium mono fluorophosphates on enamel. They concluded that a new hydroxyapatite and sodium monofluoro-phosphate containing toothpaste drastically reduced the demineralization of enamel caused by acid environment compared to control treatments. The remineralization of sub-surface enamel lesions were significantly increased compared to a non-fluoride toothpaste. An invivo radiolabelled hydroxyapatite study has evidenced that calcium ions from the hydroxyapatite are available to be involved in enamel remineralization processes. These calcium ions can help protecting from dental erosion and dental caries.

***Jing et al (2010)*[19]** used an in-vitro pH cycling model to evaluate the effect of nano-hydroxyapatite on remineralization of artificial root caries. Healthy human teeth fragments taken from the cervical portion of the root were immersed in a demineralization

solution. Nano-hydroxyapatite positively affected the demineralized surface as revealed by Scanning electron microscopy images. In accordance with this study, nano-hydroxyapatite can be a promising agent for non – invasive root caries therapy for artificial root caries lesions.

Itthagarun et al (2010)[15] tested whether the modified in-vitro pH-cycling model would work successfully as a means of investigating the de-/re-mineralization effects of nanoparticle hydroxyapatite toothpaste. They compared such effects of nanoparticle hydroxyapatite toothpaste with those of sodium fluoride toothpaste. It was revealed that progression and mineral changes in initial enamel lesions can be evidenced successfully using the modified in-vitro pH-cycling model. There was reduction in the rate of lesion progression with the application of both the 10% hydroxyapatite and the 950ppm sodium fluoride toothpastes in comparison with application of toothpaste without either active ingredient.

Kidd Edwina (2010)[22] assembled the biologic evidence behind caries removal and which would rather uncomfortably challenge traditional teaching. They brought out that partial caries removal in

asymptomatic primary or permanent teeth decreases the pulpal exposure risk.Further randomized control clinical investigations will be needed to eliminate demineralized tissue before restoring teeth.Particularly, the use of the technique in approximal lesions needs special investigation & the probability of an adverse effect on the material should be assessed.

Gonz_alez-Cabezas carlos et al (2010)[9] discussed about the caries chemistry by describing the events of remineralization and demineralization with relevance to clinical scenario. They ascertained that dental caries lesions develop when demineralization of the tooth surface exceeds the mineral transactions that occur regularly between crystals at the tooth surface and the surrounding environment. From the mechanistic point of view, these initial events hold importance. Until early demineralization becomes clinically visible or detectable; it does not hold any relevance from the clinical point of view. This clinical threshold will certainly change when newer and more sensitive detection instruments become available. When favourable conditions for lesion progression continue for a significant time, these initial noncavitated lesions will continue to progress. Finally, the lesion surface collapses leading to cavity formation.

Hara et al (2010)[11] discussed about the environment of caries. The potential to develop caries lesions happens only when the tooth surfaces are covered with biofilm. The multifaceted caries process makes the discussion of any individual component difficult, precluding the major etiologic factor, which is the dental biofilm. The pathogenicity of the dental biofilm is modified by the interaction of the saliva, the acquired pellicle, the diet and the tooth structure itself.

Fontana et al (2010)[8] overviewed the caries disease process that can provide the attention into the world of evidence-based caries management. The authors emphasised the need to keep updating in this evidenced based field. since the time of pre-Neolithic humans (10,000 BC) caries prevalence was reported between 1.4% and 12.1% of carious teeth. The ‘‘diagnosis’’ of dental caries depends on objective determination of whether the lesion is present at one point in time and a characterization of how severe it is when it has been detected. Most importantly, to assess if it is active or arrested, assimilation of all available data by a human professional is needed. One of the prominent factors for caries risk assessment and management and decision making should be this diagnosis.

Buzalaf et al (2010)[2] reviewed the current literature on existing pH-cycling models for the *in-vitro* evaluation of the efficacy of fluoridated dentifrices for caries control, focusing on their strengths and limitations. The primary outcome expected in remineralization models was the decrease of demineralization or the increase of remineralization as measured by different methods or tooth fluoride uptake. This critical review of literature showed that the ability of currently available pH-cycling models to evaluate dose-response and pH-response of dentrifices in particular fluoride dentifrices. They also evaluated the response of new active principles on the efficacy of fluoridated dentifrices, as well as their relation with other anti-caries modalities.

Twetman et al (2010)[41] discussed on the non-fluoride management of the caries disease process. They described the process of caries as a dynamic balance between re- and demineralization. The caries lesion occurs when more minerals are lost than that are gained from the hard tissues over time. Generally, bacteria which are members of the commensal micro flora secrete acids will dissolve the dental hard tissues. The highly acid-producing and acid tolerating species of bacteria have an ecological advantage, during periods of

constant pH stress. This can amount to a simplification of the biofilm diversity. The key to newer approach to caries is to counteract rapid decreases in pH in the dental biofilm, along with maintaining a neutral environment that can facilitate microbial homeostasis.

***Najibfard et al (2010*)[25]** in their situ study investigated the efficacy of nano-hydroxyapatite dentifrices on (1) remineralization of early caries lesions and (2) inhibition of demineralization of sound enamel surface. They reached the conclusion that nano-hydroxyapatite dentifrice caused remineralization of initial caries lesions comparable to fluoride dentifrice, thereby inhibiting caries progression. They suggested that nano-hydroxyapatite dentifrice can be an effective alternative to fluoride toothpaste.

***Peters et al (2010)*[31]** proposed non-invasive strategies for repair of demineralized tissue. Traditionally, caries lesion management was mainly by operative treatment. Now, the focus is on oral biofilm balance rebalancing the interplay between demineralization and remineralization therapies. A more porous structure results when caries lesions develop by mineral dissolution from the tooth tissues. The management that focus to rebalance between demineralization and remineralization always tip the balance towards an increasing

mineral uptake in the tissue. This will result in repair of the damage and also prevent new lesions from forming consecutively.

Hurlbutt et al (2010)[14] described dental caries is a pH disease. The pH of saliva plays a critical role in development and progress of dental caries by acting as a selective factor for cariogenic biofilm. According to science it is pH, more than sugar, contributing as the selective factor for cariogenic plaque biofilms. Low salivary pH curtails the hostile environment for protective oral bacteria and favours the growth and proliferation of acidogenic bacteria. This can account for an environmental balance shift to promote cariogenic bacteria. Chemistry points out at what pH enamel and dentin will demineralize. Thus it is possible to change the plaque biofilm, remineralize existing lesions, and prevent the disease altogether by controlling pH.

Haghgoo et al (2011)[10] studied on the remineralization potential of primary carious lesion. The primary micro hardness value of each tooth was measured, before immersing them in 40 ml soft beer. Consequently, secondary micro hardness values were also evaluated. The tertiary micro hardness values were measured after randomly placing the teeth in two groups-nano-hydroxyapatite

solution and drinking water. The results of the present study revealed that nano-hydroxyapatite solution has the remineralization potential. It remineralizes enamel which was eroded by soft beer.

Karlinsey et al (2011)[20] used microhardness and fluoride uptake analyses to evaluate the remineralization effects of four dentifrice systems in-vitro. A 10-day pH cycling model was used to test the remineralization potential of the white-spot lesions in bovine and human enamel. The specimens were subjected to analysis for surface microhardness, enamel fluoride uptake, and cross-sectional microhardness. The cross-sectional micro hardness measurements after pH cycling revealed similar profiles for both human and bovine enamel. It was also observed that human enamel was more sensitive than bovine enamel to 500 ppm of fluoride uptake. The inherent structural differences between the human and bovine enamel may be the reason for this apparent sensitivity.

Tschoppe et al (2011)[40] studied remineralization effect of nano-hydroxyapatite toothpastes on bovine enamel and dentine subsurface lesions in-vitro. They observed that the nano-hydroxyapatite containing toothpastes revealed higher remineralizing

effects as compared to amine fluoride toothpastes on bovine dentine. The remineralization effect on enamel showed comparable trends.

Itthagarun et al (2011)[16] investigated and concluded that fluoridated milk has re-mineralising efficacy on early enamel lesions. They also tested whether increasing fluoridated milk volume has any effect on its re-mineralising efficacy. They concluded that 2.5ppm fluoride concentration in milk provided remineralization potential similar to that of milk with higher fluoride concentration.

Ramli et al (2011)[32] described Hydroxyapatite as a desirable implant material due to its biocompatibility and osteoconductive properties. They devised a practical and economical approach to produce pure Nano porous Hydroxyapatite using a Hydroxyapatite-chitosan template. The Hydroxyapatite containing nano size pores were obtained from the removal of chitosan through calcination. Disordered interconnected pores were revealed in surface morphology analysis performed on both uncalcined and calcined Hydroxyapatite-chitosan. A higher surface area and more even distribution of Hydroxyapatite nanoparticles resulted due to the removal of chitosan. The final material consisted of highly crystalline

noncarbonated hydroxyapatite as confirmed by both X-ray diffraction and Fourier transform infrared analyses.

Zero et al (2011)[48]studied about the dental caries and its consequences. The fluoride usage in developed countries, has led to a decline in the prevalence, severity and caries progression rate. The new trend in dental caries management includes early stage caries diagnosis, risk assessment, with interventional strategies to establish prognosis focusing on reversal of the carious process.

MATERIALS USED

1. Freshly extracted human central incisors
2. Acetic acid -{MERCK SPECIALITIES}
3. Calcium nitrate-{MERCK SPECIALITIES}
4. Potassium dihydrogen orthophosphate

   –{HIMEDIA LABORATORIES}
5. Sodium fluoride-{S.D.F.CHEMICALS}
6. HEPES-(20mM/l(4-(2-hydroxyethyl)-1-piperazine-ethane-sulfonicacid)-{SISCO RESEARCH LABORATORIES}
7. Calcium chloride-{MERCK SPECIALITIES}
8. Potassium chloride-{MERCK SPECIALITIES}
9. Potassium hydroxide-{MERCK SPECIALITIES}
10. SodiumAzide-{MERCK SPECIALITIES}
11. Distilled water-{MERCK SPECIALITIES}
12. Nano-hydroxyapatite powder (mKNANO IMPEX CORP,CANADA)

13. Hydrochloric acid -{MERCK SPECIALITIES}
14. Polymethylmethacrylate resin
15. 40 polypropylenevials

ARMAMENTARIUM

16. Diamond discs
17. Fine grit silicon carbide discs (1200 grit)-{3M ESPE}
18. Carborundum discs
19. Vernier caliper-{AEROSPACE DIAL CALIPER}
20. PH meter-{ELICO}
21. Cutting lathe-{SUGUNA}
22. Weighing balance-{K RAJ INSTRUMENTS}
23. Micro motor with straight angled hand piece. [NSK]

SPECIAL EQUIPMENTS USED

24. Vickers microhardness tester-{MH6}

25. Incubator-{ASHOK UNITED SCIENTIFIC COMPANY}

26. Atomic force microscope{XE-70 PARK SYSTEMS}

METHODOLOGY

A total of 40 intact single rooted human central incisors which are devoid of cracks, caries and white spot lesions were collected and cleaned ultrasonically to remove calculus and tissue debris and stored in water containing 0.1% thymol until needed for the study.

Enamel block preparation

The teeth were decoronated with diamond disc and enamel blocks of size 4mmx4mm were prepared after measuring with vernier caliper. These blocks were embedded in polymethylmethacrylate resin. The labial enamel surface was ground flat with carborundum discs and polished with fine grit (1200 grit) silicon carbide discthere by removing approximately 100 μm of the outermost enamel layer to prepare a flat surface.

Baseline microhardness test

Baseline surface hardness of the sound enamel after polishing was done with a Vickers microhardness indenter at 200 gf load for 15s[20]. Enamel blocks showed baseline surface microhardness (SMH) between 270.2 and 320.0 vickers hardness numbers (VHN)

which coincided with the microhardness value of normal human enamel.

Solution preparation

Demineralization solution: The demineralization solution was used, to prepare artificial caries like lesions in the enamel and in the pH cycling. The solution used contained 2.2mM Calcium nitrate ($Ca(NO_3)_{2)}$, 2.2mM Potassium dihydrogen orthophosphate (KH_2PO_4) and 0.1 ppm sodium fluoride (NaF). This solution was adjusted to a pH 4.5 with acetic acid (50mM) solution using pH meter.

***Remineralization solution*:** The remineralization solution used in pH-cycling contained 20mmoll−1 l(4-(2-hydroxyethyl)-1-piperazine-ethane-sulfonicacid) HEPES, 1.5mM Calcium chloride (CaCl2), 0.9 mM Potassium dihydrogenortho phosphate (KH_2PO_4)), 130mM Potassium Cholride (KCl) and 1 mM sodium Azide (NaN_3). The pH was adjusted to 7.0 with potassium hydroxide (KOH) using a pH meter. These solutions were in accordance to those used by Ten Cate and Duijsters. The nano-HA crystals were of 20-40 nano-metergrade (mK Nano-Impex corporation Canada) which were similar in crystallinity to apatitein bone and enamel. Treatment

solutions were 1000 ppm sodium fluoride aqueous (Positive control), distilled water (Negative control); 5%, 8% and 10%wtnano-HA suspension liquid in distilled water, pH adjusted to 7.0 using 2 M HCL.

Preparation of early artificial enamel caries lesions

Early artificial enamel lesions were produced according to Ten Cate and Duijsters. Each specimen was immersed in 8 ml of demineralization solution for 72 h at 37˚C. After artificial caries preparation, the surface microhardness of the enamel blocks was again measured (SMH 1). When baseline measurements were made, indentations were spaced at 100 μm from each other. After artificial caries preparation, baseline Vickers hardness number values (SMH 1) were between 20.3 and 40.1 and were selected for pH-cycling.

pH-cycling model

The specimens were randomly divided into five groups (8 specimens/group) according to the treatment solutions.

Group I: 1000ppm sodium fluoride (Positive control)

Group I: Distilled water (Negative control)

Group III: 5 wt% Nano-hydroxyapatite suspension liquid in distilled water

Group IV: 8 wt% Nano-hydroxyapatite suspension liquid in distilled water

Group V: 10 wt% Nano-hydroxyapatite suspension liquid in distilled water

The cycling schedule was designed to simulate the pH dynamics of the oral environment .The regime has been reported by White.[46] The demineralization and remineralization cycles consisted of the episodes shown in TABLE 1. Each cycle involved 2 hours of demineralization in order to simulate the daily acid challenges occurring in the oral cavity. The regimen was repeated for 12 days and temperature maintained at 37˚C in an incubator. The demineralizing and remineralizing solutions were freshly made every third day where as the treatment solutions were made daily.

Surface microhardness analysis:

The enamel blocks were subjected to surface microhardness evaluation (SMHn) after 3, 6, 9 and 12 days of application. Five indentations were placed next to the previous measurement at100 μm intervals at each time point. The comparison of mean values of all

five measurements at different application times were done. The percentage surface microhardness recovery was calculated as [after *n*(th) days % SMHR = 100 (SMH *n*–SMH 1)/(SMH–SMH 1)] (*n* =3/6/9/12).

Atomic force microscopic examination

Three dimensional atomic force microscopic images were taken before and after remineralization to evaluate surface roughness parameters. The surface parameters taken in to consideration were Rrms and Rz. The root mean square roughness (Rrms) represents the height distribution relative to the mean line. Rz describes the average maximum peak-to-valley height of five consecutive sampling depths.

Five fields were used to measure the surface roughness on every window with measuring area 1000nmx1000nm, and the intervals of each other are 100 mm. The surface roughness of all the lesions on the window were analyzed, and then the mean values of parameters were calculated. The mean values of all the measurements before and after the remineralization test were then compared, the change percentage of, Rrms and Rz was calculated as follows:

$\%DRrms=(Rrms_2-Rrms_1)/Rrms_1x100$

$\%DRz=(Rz_2-Rz_1)/Rz_1x100$[49]

METHODOLOGY -OVERVIEW

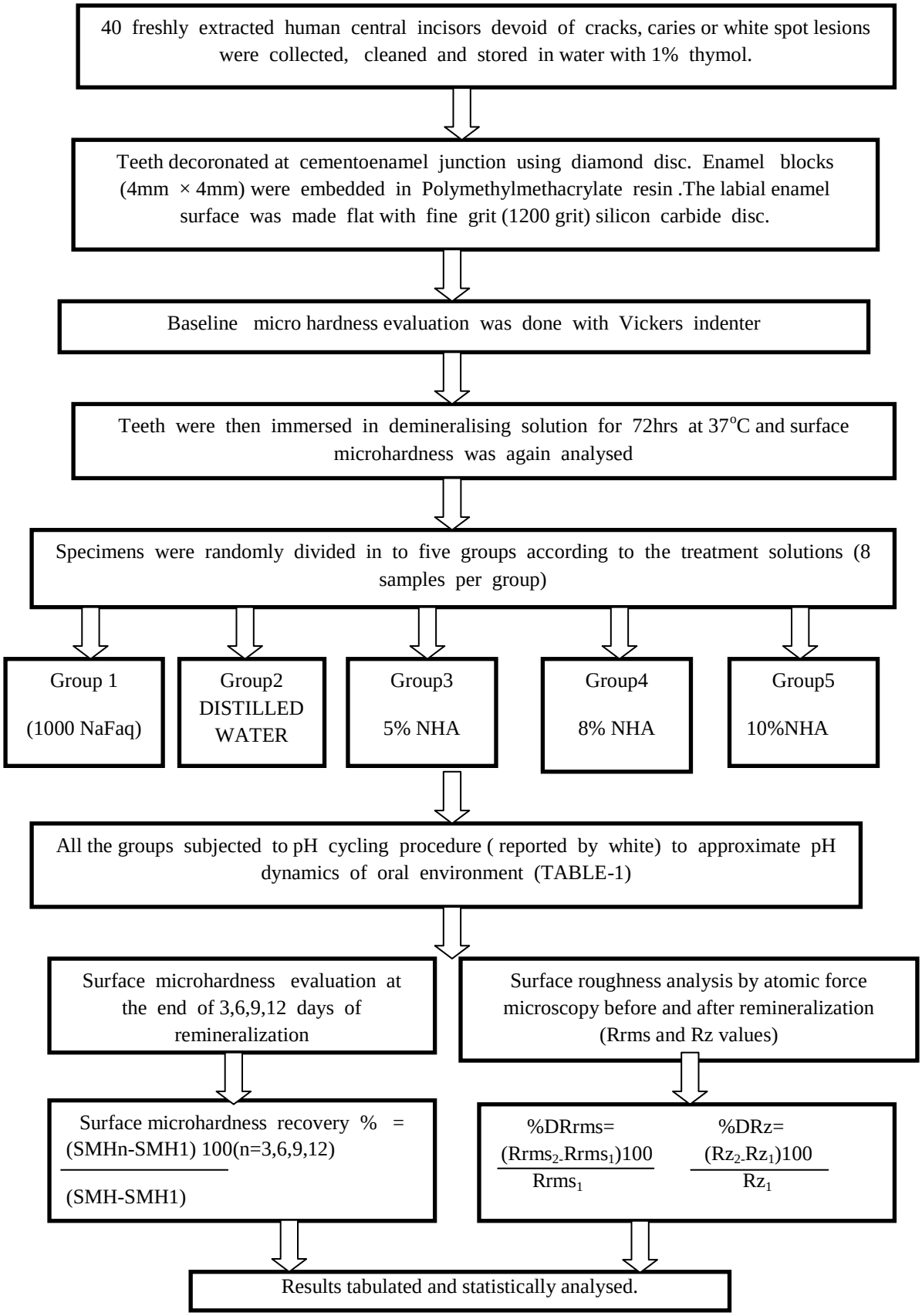


Fig.1: TEETH SAMPLES

Fig.2: ARMAMENTARIUM

Fig.3: DECORONATION OF TOOTH

Fig.4: CUTTING LATHE

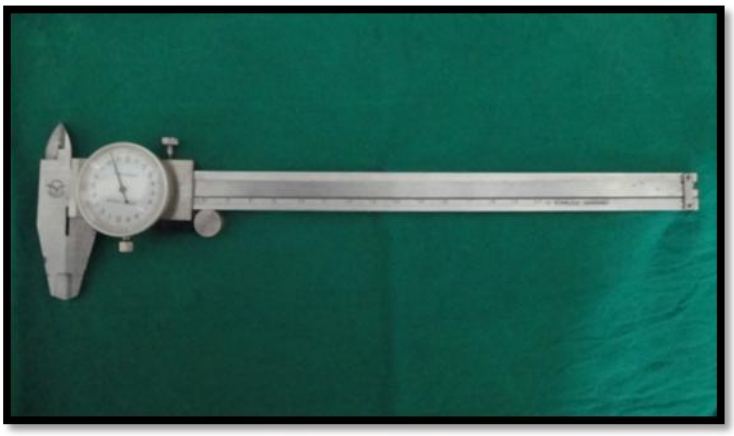


Fig.5: VERNIER CALIPER

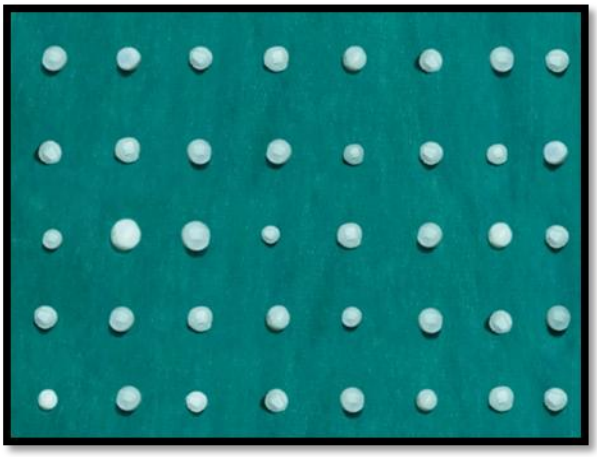


Fig.6: ENAMEL BLOCKS EMBEDDED IN POLYMETHYLMETHACRYLATE RESIN

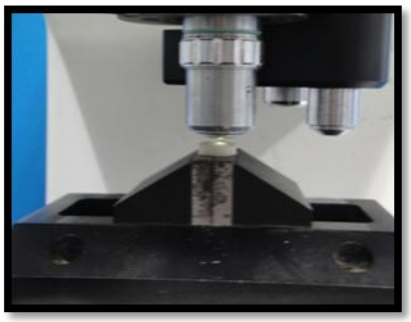


Fig.7: VICKERS MICROINDENTER

Fig.8: NANO-HYDROXYAPATITE POWDER

Fig.9: POWDERS FOR REMINERALIZING SOLUTION

Fig 10: POWDERS FOR DEMINERALIZING SOLUTION

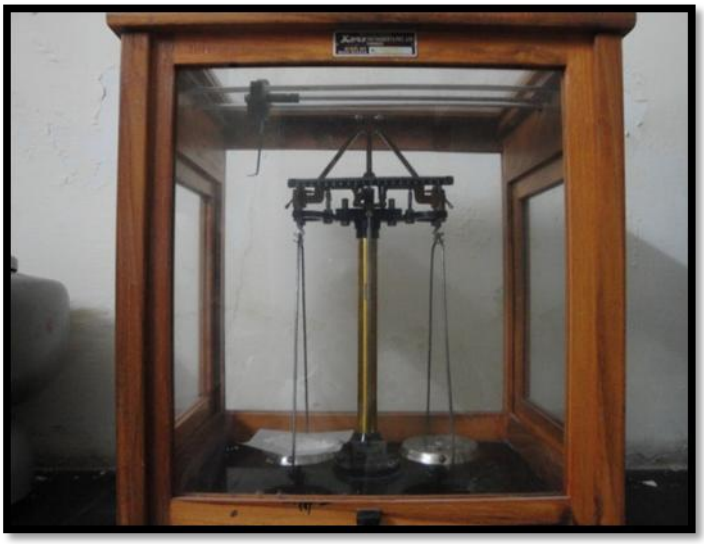


Fig.11: WEIGHING BALANCE

Fig.12: PH METER

Fig.13: PREPARED TREATMENT SOLUTIONS

Fig.14: INCUBATOR

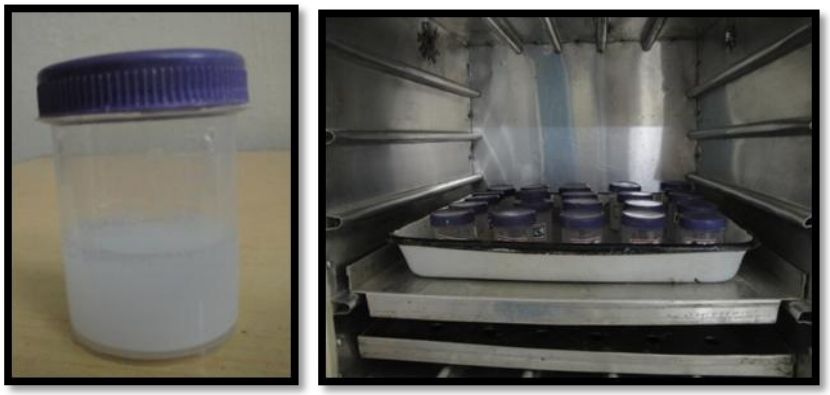


Fig.15: SAMPLES KEPT IN THE INCUBATOR

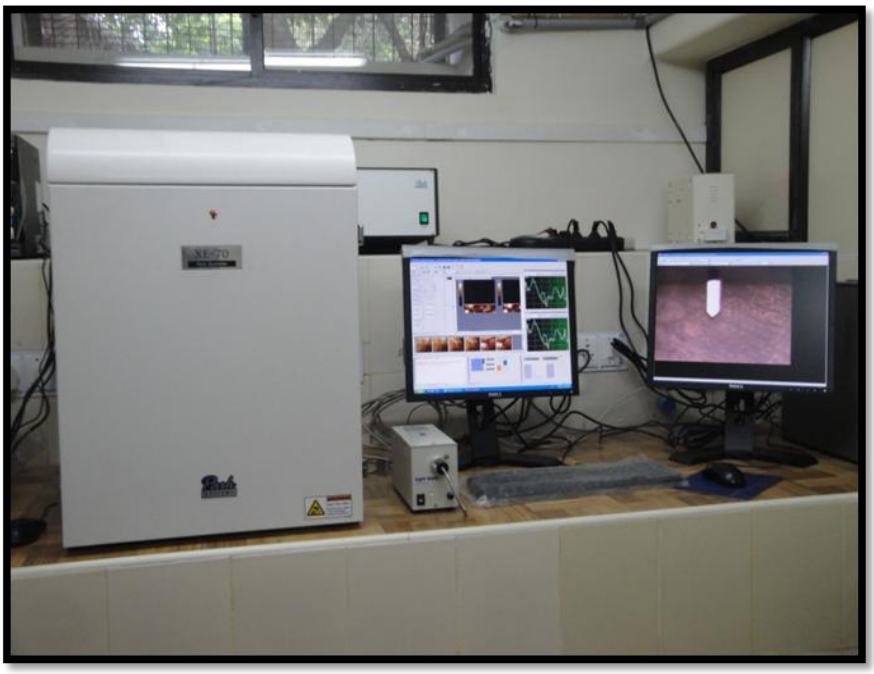


Fig.16: ATOMIC FORCE MICROSCOPE

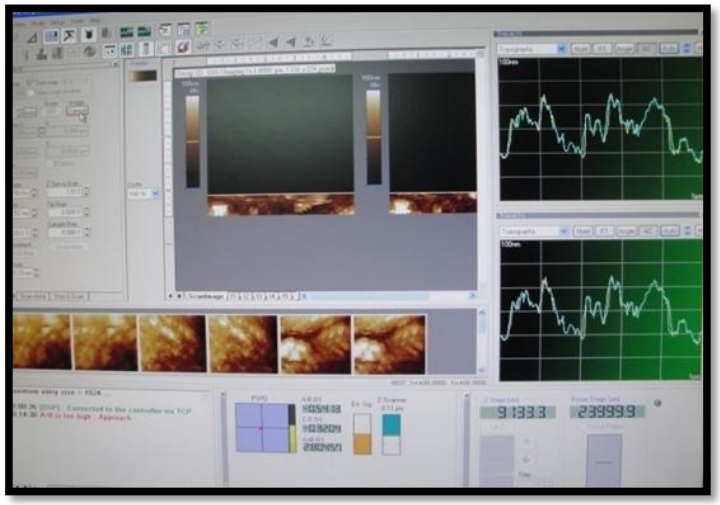


Fig.17: ATOMIC FORCE MICROSCOPE MONITOR

TABLE-1: THE PH-CYCLING MODEL IN THE EXPERIMENT

Time	Experimental solution
8:00 a.m.–8:03 a.m.	Treatment solutions
8:03 a.m.–9:00 a.m.	Remineralization solution
9:00 a.m.–9:03 a.m	Treatment solutions
9:03 a.m.–11:00 a.m	Remineralization solution
11:00 a.m.–1:00 p.m	Demineralization solution
1:00 p.m.–3:00 p.m.	Remineralization solution
3:00 p.m.–3:03 p.m	Treatment solution
3:03 p.m.–4:00 p.m.	Remineralization solution
4:00 p.m.–4:03 p.m	Treatment solutions
4:03 p.m.–8:00 a.m.	Remineralization solution

The treatment solutions, remineralization solution and demineralization solution were used as described above.

TABLE-2: SURFACE MICROHARDNESS RECOVERY (SMHR) ANALYSIS OF ENAMEL BLOCKS AT VARIOUS TIME PERIODS ACCORDING TO THE TREATMENTS BY ANOVA AND POST-HOC TUKEY TESTS

SOLUTIONS	BASELINE	BEFORE PH-CYCLING	SMHR-3DAYS	SMHR-6DAYS	SMHR-9DAYS	SMHR-12DAYS
POSITIVE CONTROL	298.93±8.22	27.59± .39[a]	29.49±.71[e]	51.29±1.21[e]	58.77±1.17[e]	63.41±1.23[e]
NEGATIVE CONTROL	297.04±6.54	29.83±1.66[ab]	2.26±.78[a]	6.75±1.08[a]	6.92±1.29[a]	7.15±1.24[a]
5%NHA	300.93±5.39	33.01±3.71[b]	14.97±.93[b]	21.78±.90[b]	27.37±1.04[b]	28.67±1.28[b]
8% NHA	300.26±5.65	31.70±2.88[ab]	22.04±.86[d]	43.14±1.22[c]	45.21±1.28[c]	46.78±1.58[c]
10% NHA	303.24±6.05	30.84±2.13[ab]	17.93±1.28[c]	47.66±1.94[d]	50.04+1.99[d]	53.62±3.52[d]
P-VALUE	0.408	0.018*	<0.001**	<0.001**	<0.001**	<0.001**

Note: 1. P-VALUE ** denotes significance at 1% level.

2. P-VALUE * denotes significance at 5% level.

3. Different alphabet between solution denotes significance at 1% level .(To distinguish the similarities in the value).

TABLE-3: COMPARISON OF MICROHARDNESS VALUES BETWEEN BASELINE AND VARIOUS TIME INTERVALS FOR POSITIVE CONTROL USING PAIRED T-TEST

GROUP	DAYS(MEAN±SD)				
	BEFORE PH-CYCLING	3 DAYS	6 DAYS	9 DAYS	12 DAYS
POSITIVE CONTROL	27.58±4.83	107.58±4.64	166.71±3.95	187.01±3.69	199.58±3.65
P VALUE	<0.001**	<0.001**	<0.001**	<0.001**	<0.001**

Note: 1. Baseline value: 298.925±8.2177

2. P-VALUE ** denotes significance at 1% level

TABLE-4: COMPARISON OF MICROHARDNESS VALUES BETWEEN BASELINE AND VARIOUS TIME INTERVALS FOR NEGATIVE CONTROL USING PAIRED T-TEST

GROUP	DAYS(MEAN±SD)				
	BEFORE PH-CYCLING	3 DAYS	6 DAYS	9 DAYS	12 DAYS
NEGATIVE CONTROL	29.82±1.6	35.86±3.04	47.82±1.82	48.26±2.56	48.88±2.46
P VALUE	<0.001**	<0.001**	<0.001**	<0.001**	<0.001**

Note: 1,Baseline value: 297.038±6.53

2. P-VALUE ** denotes significance at 1% level

TABLE-5: COMPARISON OF MICROHARDNESS VALUES BETWEEN BASELINE AND VARIOUS TIME INTERVALS FOR 5%WT NHA USING PAIRED T-TEST

GROUP	DAYS(MEAN±SD)				
	BEFORE PH-CYCLING	3 DAYS	6 DAYS	9 DAYS	12 DAYS
5%NHA	33.01±3.70	73.11±5.13	91.38±5.64	106.35±5.67	109.82±5.77
P-VALUE	<0.001**	<0.001**	<0.001**	<0.001**	<0.001**

Note: 1,Baseline value: 300.925±5.39

2,P-VALUE ** denotes significance at 1% level

TABLE-6: COMPARISON OF MICROHARDNESS VALUES BETWEEN BASELINE AND VARIOUS TIME INTERVALS FOR 8%WT NHA USING PAIRED T-TEST

GROUP	DAYS(MEAN±SD)				
	BEFORE PH-CYCLING	3 DAYS	6 DAYS	9 DAYS	12 DAYS
8%NHA	31.7±2.87	90.862±3.39	147.525±3.25	153.06±2.26	157.275±2.93
P VALUE	<0.001**	<0.001**	<0.001**	<0.001**	<0.001**

Note: 1, Baseline value: 300.263±5.64

2, P-VALUE ** denotes significance at 1% level

TABLE-7: COMPARISON OF MICROHARDNESS VALUES BETWEEN BASELINE AND VARIOUS TIME INTERVALS FOR 10%WT NHA USING PAIRED T-TEST

GROUP	DAYS(MEAN±SD)				
	BEFORE PH-CYCLING	3 DAYS	6 DAYS	9 DAYS	12 DAYS
10%NHA	30.83±2.12	79.63±2.86	160.61±2.82	167.07±3.08	176.77±6.87
P VALUE	<0.001**	<0.001**	<0.001**	<0.001**	<0.001**

Note: 1, Baseline value: 303.238±6.05

2, P-VALUE ** denotes significance at 1% level

TABLE-8: THE SURFACE ROUGHNESS PARAMETER RRMS OF CARIOUS ENAMEL BEFORE AND AFTER REMINERALIZATION WITH AFM MEASUREMENTS BY ANOVA AND POST-HOC TUKEY TESTS

Groups	Rrms1	Rrms2	%DRrms
Positive control	84.15±2.84[a]	58.78±3.00[a]	-30.14±2.88[a]
Negative control	93.91±2.83[b]	112.07±5.81[c]	19.38±6.19[d]
5% NHA	84.34±1.44[a]	74.29±1.42[b]	-11.91±.56[c]
8% NHA	85.76±1.87[a]	70.23±3.10[b]	-18.07±4.22[b]
10% NHA	83.28±1.77[a]	63.27±2.45[a]	-24.02±2.71[ab]
p-value	<0.001**	<0.001**	<0.001**

Note: P-VALUE ** denotes significance at 1% level

TABLE-9: THE SURFACE ROUGHNESS PARAMETER Rz OF CARIOUS ENAMEL BEFORE AND AFTER REMINERALIZATION WITH AFM MEASUREMENT BY ANOVA AND POST-HOC TUKEY TESTS

Groups	Rz1	Rz2	%DRz
Positive control	414.02±2.56[a]	274.92±1.95[a]	-33.59±.77[a]
Negative control	475.82±2.71[c]	555.07±3.50[e]	16.66±.93[e]
5%NHA	416.50±3.34[ab]	370.25±1.55[d]	-11.10±.75[d]
8% NHA	420.20±1.65[b]	339.98±2.03[c]	-19.09±.50[c]
10% NHA	417.19±1.29[ab]	290.21±2.04[b]	-30.44±.56[b]
p-value	<0.001**	<0.001**	<0.001**

Note: P- VALUE ** denotes significance at 1% level

RESULTS

The results of the present study were subjected to statistical analysis to interpret the significant differences in surface microhardness recovery, Rrms & Rz values within each group and also between the groups using one way ANOVA and POST HOC TUKEY tests. Paired t-tests were also used to compare the microhardness at baseline and at the end of various time intervals for different groups.

One way analysis of variance (ANOVA) is used to study the overall variance within groups. The mean surface microhardness recovery percentage and standard deviation were calculated for each treatment groups at various time intervals. The Post hoc Tukey test was used for intra group comparison in order to determine which groups differ from each other. The Post hoc Tukey Test is designed to perform a pair wise comparison of the means to identify the specific sub groups in which significant difference expression occurs. Paired ‘t’-test is applied to unpaired data of independent observation made on individuals of two different or separate groups or samples drawn from two populations.

TABLE 2 represents percentage of surface microhardness recovery (%SMHR) analysis of enamel blocks at various time periods by ANOVA. It revealed remarkable remineralization of the enamel blocks in all treatment groups except distilled water (Negative control) in the pH cycling experiment. Remineralizing effect of positive control (Sodium fluoride) was high where p-value was statistically significant. ($P<0.001$). In the present study, among the various concentrations of nano-hydroxyapatite tested 10% wt NHA showed the best results ($P<0.001$). Percentage of surface microhardness recovery (%SMHR) increased with nano-hydroxyapatite concentration at each time point in the pH-cycling after 3,6,9,12 days with a significant p-value in the experiment.($P<0.001$). The highest % of surface microhardness recovery was observed with 10%wt nano-hydroxyapatite (10% wt NHA) and the lowest was for negative control (Distilled water).The %SMHR revealed that, for all treatment groups, the remineralization rate increased significantly in the first 6 days of pH-cycling. Post-hoc Tukey tests showed that after each time interval, treatment groups showed statistically significant surface microhardness recovery.

TABLE 3, Baseline microhardness values were compared with microhardness values at various time intervals for **positive control** (sodium fluoride) by paired t-tests. It was observed that, the remineralization rate increased significantly ($P<0.001$) from the baseline in the first 6 days of pH-cycling although microhardness values increased till day 12.

TABLE 4, Baseline microhardness values were compared with the values at various time intervals for **negative control** (Distilled water) by paired t-tests. It was revealed that, the remineralization rate increased ($P<0.001$) at various time intervals compared to the baseline value of enamel blocks.

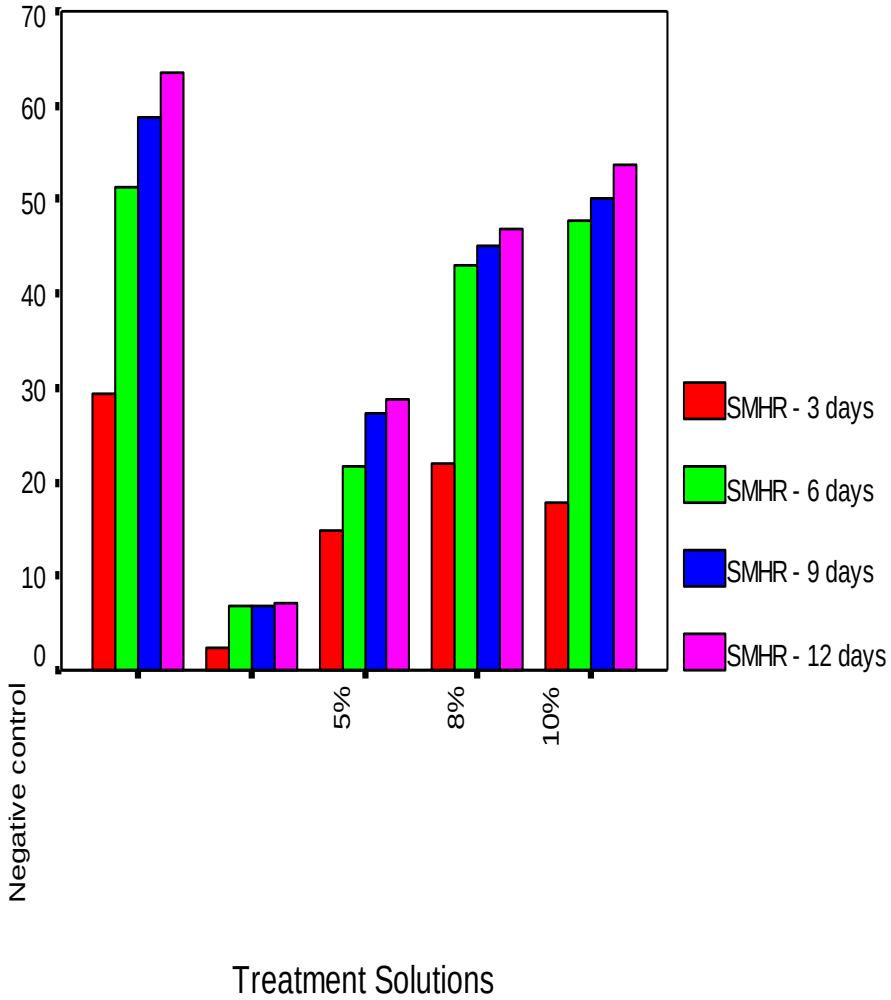
TABLE 5, 6 and 7 shows microhardness values at the baseline and at the end of each time intervals were compared for 5%wt NHA, 8%wt NHA, 10%wt NHA respectively by paired t-tests. The microhardness values showed statistically significant values for remineralization ($p<0.001$) in all these groups with greater increase from baseline value observed after 6 days.

Table 8, shows significant difference in Rrms before and after remineralization for each group ($p<0.001$), although Rrms of all the groups revealed similar values before remineralization ($p<0.001$). The

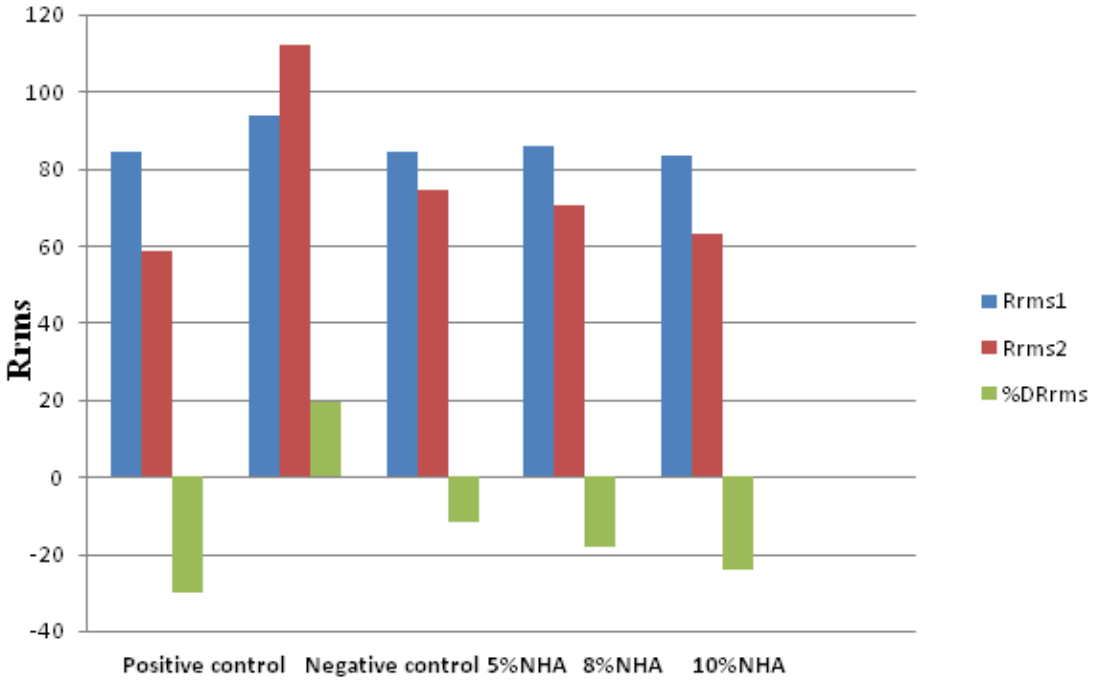
decrease of Rrms in positive control (sodium fluoride) treated group was more compared to nano-hydroxyapatite treated group ($p<0.001$); In nano-hydroxyapatite groups decrease of surface roughness was more evident in the 10%wt NHA followed by 8%wt NHA and 5%wt NHA. However, Rrms in negative control (Distilled water) showed high degree of surface roughness which was statistically significant ($p<0.001$).

Table 9, Rz of all the groups showed similar values before remineralization ($p<0.001$); after the remineralization the changes of Rz for each group were same with those of Rrms. The decrease of Rz in positive control (sodium fluoride)-treated group was more than that in nano-hydroxyapatite treated group ($p<0.001$). In nano-hydroxyapatite, 10%wt NHA showed a greater decrease followed by 8%wt NHA and 5%wt NHA. However, Rz in distilled water treated group increased significantly ($p<0.001$).

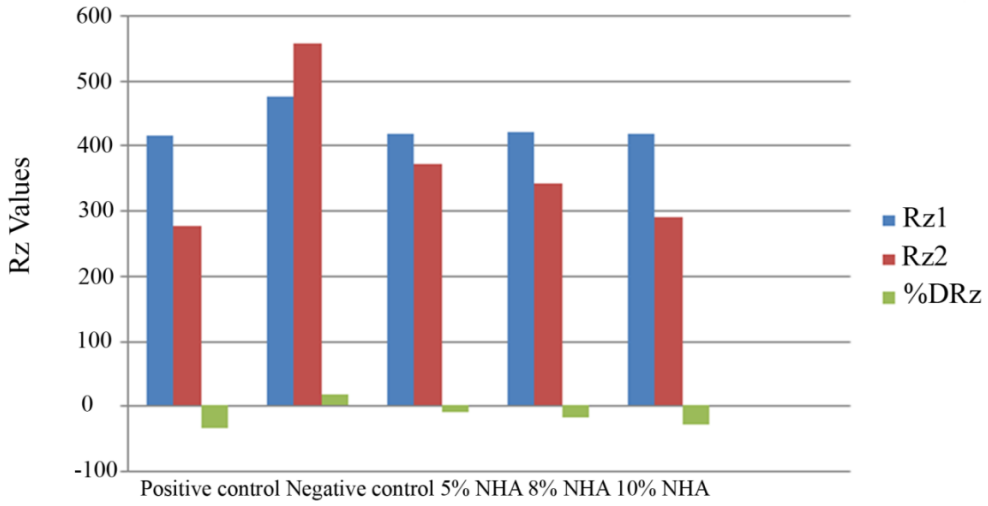
GRAPH-1: PERCENTAGE SURFACE MICROHARDNESS RECOVERY (SMHR%) OF ENAMEL BLOCKS AT VARIOUS TIME PERIODS ACCORDING TO THE TREATMENTS.



GRAPH-2: SURFACE ROUGHNESS VALUE Rrms BEFORE AND AFTER REMINERALIZATION ALONG WITH THE PERCENTAGE CHANGE OF VARIOUS TREATMENT SOLUTIONS



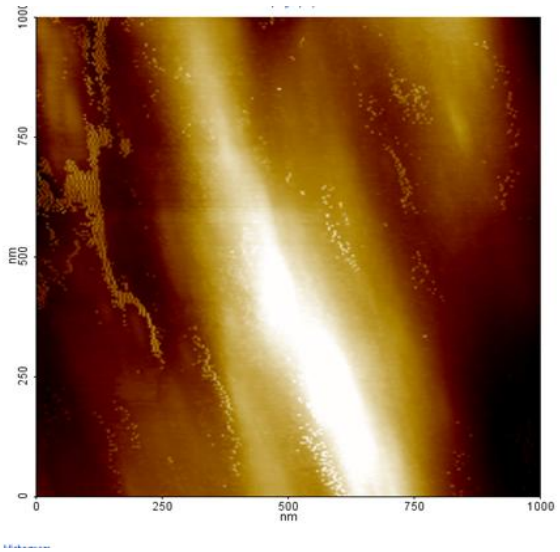
GRAPH-3: SURFACE ROUGHNESS VALUE Rz BEFORE AND AFTER REMINERALIZATION ALONG WITH THE PERCENTAGE CHANGE OF VARIOUS TREATMENT SOLUTIONS



REPRESENTATIVE ATOMIC FORCE MICROSCOPIC IMAGES

NORMAL HUMAN TOOTH

SURFACE TOPOGRAPHY-TWO DIMENSIONAL IMAGE



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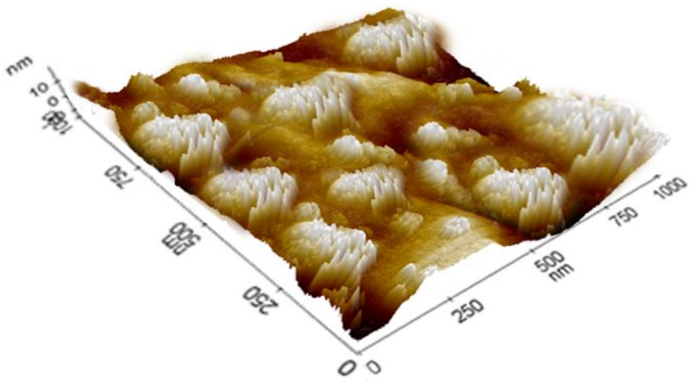
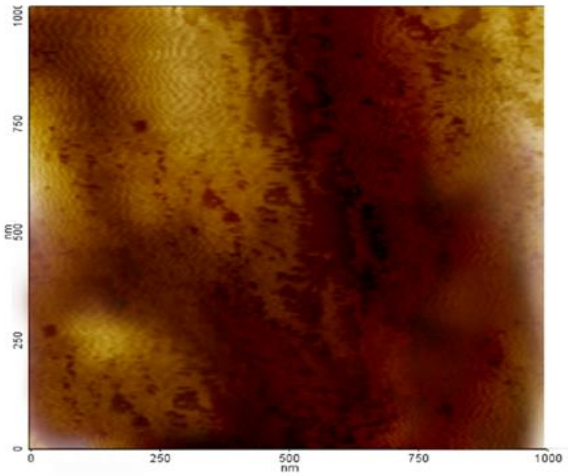


DEMINERALISED TOOTH

SURFACE TOPOGRAPHY-TWO DIMENSIONAL IMAGE



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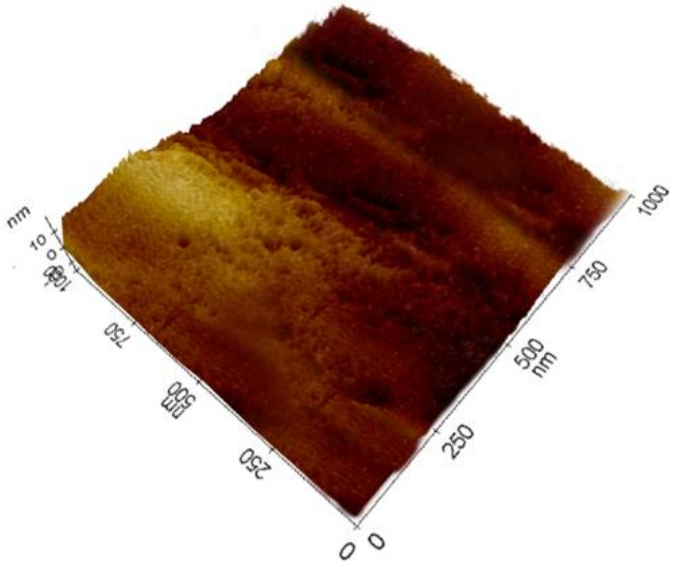

SODIUM FLUORIDE

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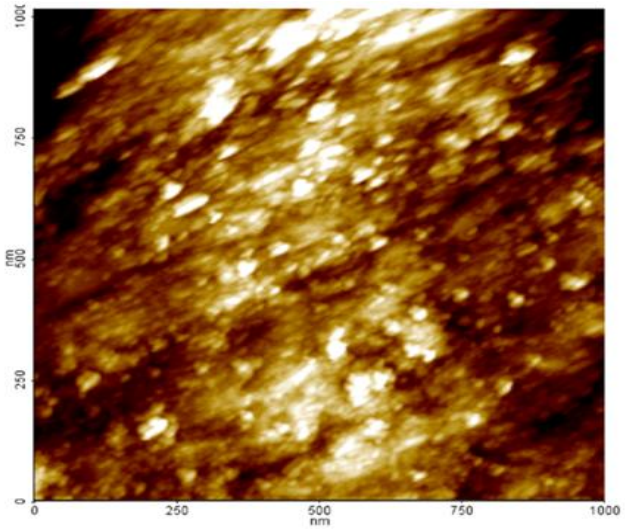

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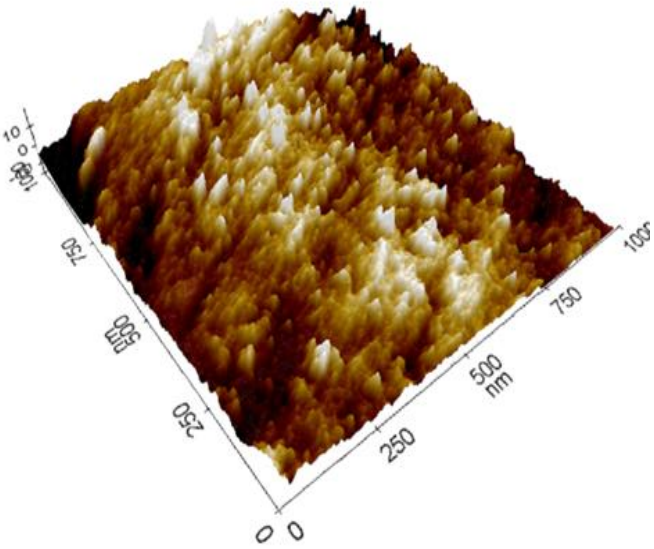

5%WT NANO-HYDROXYAPATITE

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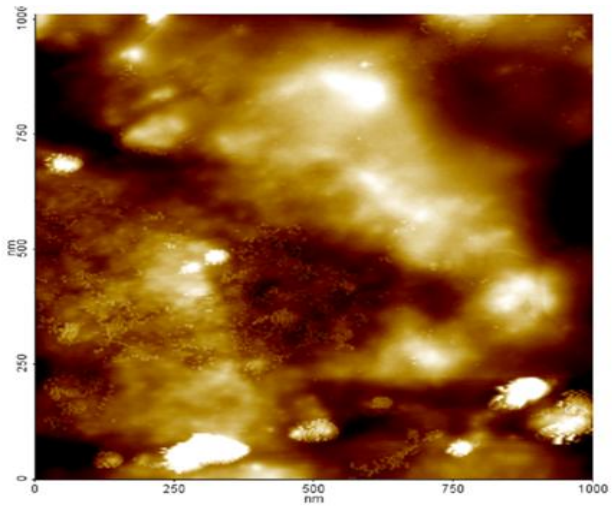

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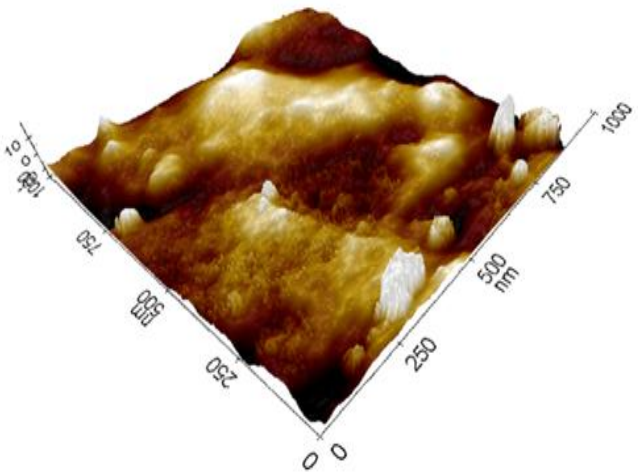

8%WT NANO-HYDROXYAPATITE

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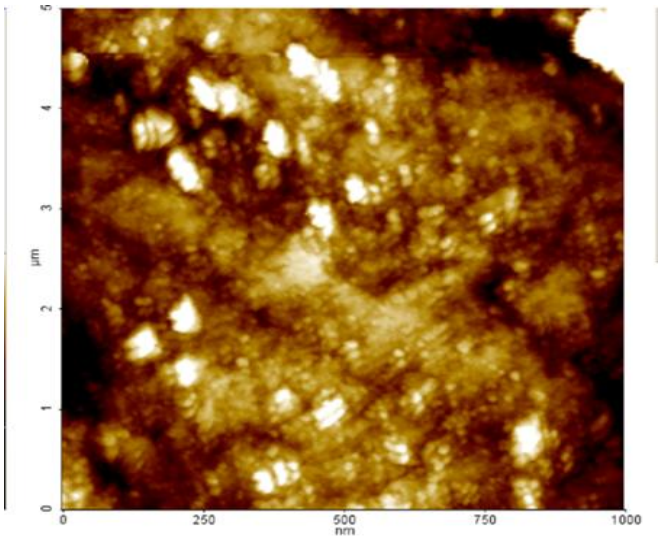

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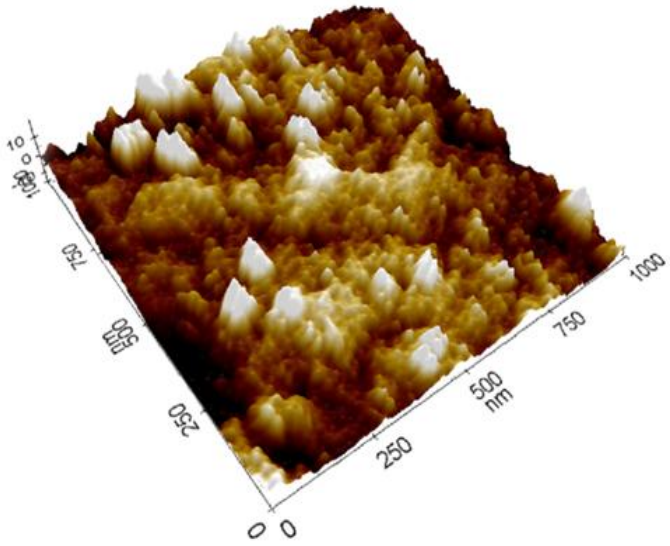
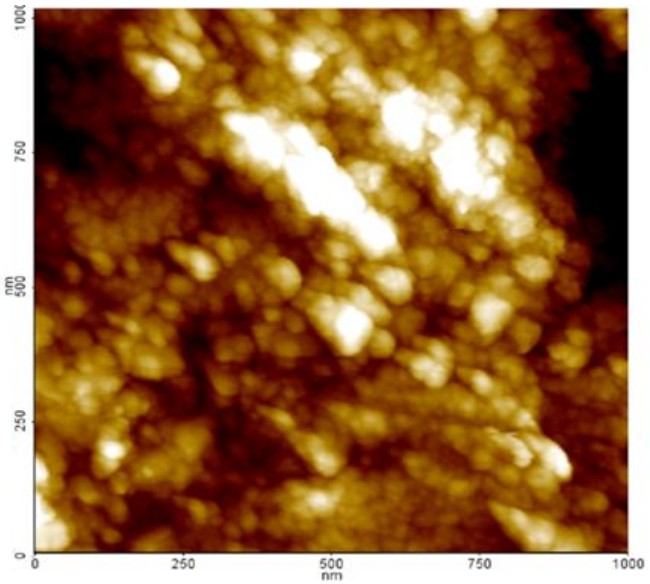
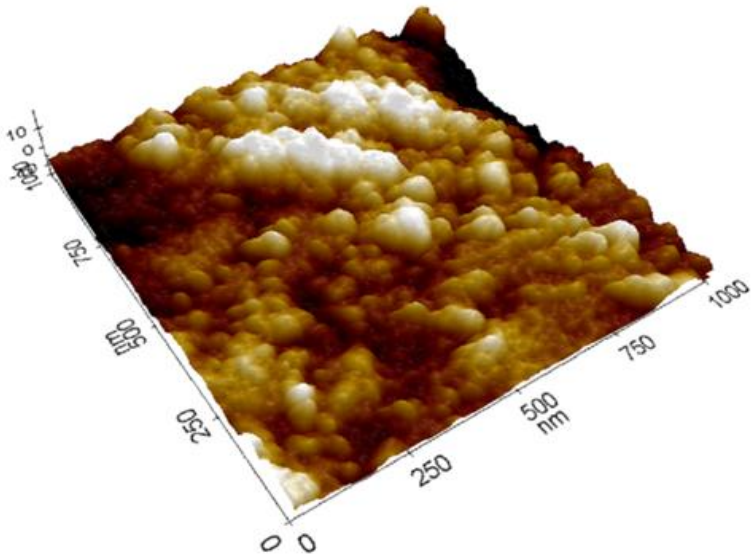


10%WT NANO-HYDROXYAPATITE

SURFACE TOPOGRAPHY-TWO DIMENSIONAL IMAGE



THREE DIMENSIONAL IMAGE



DISCUSSION

The process of dental caries is a dynamic balance between mineral loss and mineral gain, particularly in surfaces covered by undisturbed biofilms.[53] Caries lesions develop only when the balance between bacterially generated acid challenges and protective factors of saliva are disturbed. Enamel has gained a lot of attention, being at the highest risk for developing caries lesions. The mineral component of enamel is not pure hydroxyapatite, but rather a mixture of compounds including carbonated apatites.[52] These differences in mineral composition can determine the stability of enamel crystals, affecting it's solubility.[48]

The genesis of caries lesion at the enamel surface is as a white spot lesion. Nikiforuk et al has stated that, enamel being acellular and avascular, it can be regarded as ***"metabolically inert structure"***. But it can still undergo remineralization and demineralization which are important physico-chemical exchange reactions.[51] These white spot lesions can get transformed either as a brown spot lesion, a re-mineralised lesion or as a complete caries reversal.[29]

The enamel crystals covered by undisturbed biofilm can undergo mineral transactions till the biofilm can maintain conditions of under saturation and over saturation with respect to the crystals. Undersaturation occurs due to loss of phosphate and hydroxyl ions which are reacting with the hydrogen ions under lower pH conditions. In the incipient caries lesion, bacterial acids from the biofilm can penetrate through the crystalline spaces of enamel leaving a subsurface lesion. To counteract this, minerals from various sources like saliva, bacteria, calculus, calcium fluoride formulations and the tooth surface itself will come in to play.[9]

At a particular point, pH can increase to a level where supersaturation conditions can occur. During this period, partially demineralized enamel crystals can be remineralized as the tendency of the solution is to precipitate minerals. Thus it is clear that pH is the strongest determinant for saturation level, along with concentration of calcium and phosphate ions .Based on this fact, Michelle Hurlbutt et al termed caries as a "**pH mediated disease**".[14]

Caries progression occurs very slowly and intermittently in normal populations as each period of demineralization is followed by a period of rest or even remineralization. The literature reveals that,

the progression of incipient caries may take 3 months to 48 months. If we are able to intervene during this time period, remineralization is possible.[53]

Remineralization of non-cavitated lesions has been reported from early twentieth century, when demineralized enamel was observed to harden in the presence of saliva. The immersion of white spot lesions in super saturated solutions of calcium and phosphate, eventually leading to it's reversal has been well documented in the literature.[29]

Among the numerous remineralizing agents, the cariostatic efficacy of fluoride has been convincingly demonstrated by Brambilla et al.[1] The fluoride, when it is incorporated in to enamel minerals forms fluorapatite or fluorhydroxyapatite there by reducing enamel solubility.[31] The assumption of beneficial effect of fluoride as mainly pre-eruptive has been changed.[16] Evidences points out that post eruptive cariostatic effect of fluoride is through the topical effect which includes:1, Inhibition of demineralization 2, Enhancement of remineralization 3, Inhibition of bacterial activity in the plaque. 4, Interference of pellicle and plaque formation.[45]

The recent studies have demonstrated that the provision of the dissolved fluoride is the key element to successful therapy. On topical application, calcium fluoride layer is formed which dissolves readily to release fluoride. The end products can be fluorapatite crystals or calcium fluoride incorporated on the tooth surface. This calcium fluoride layer can act as a reservoir of fluoride ions promoting remineralization. Thus the constant low levels of topical fluorides have a profound effect in creating a more caries resistant enamel through repeated cycles of demineralization and remineralization.[33]

Along with the time tested fluoride, many attempts have been made to remineralize white spot lesions. Technologies available for remineralization include Caesinphosphopeptide-Amorphous calcium phosphate, Tricalciumphosphate, Novamin, Enamelon, Dicalcium-phosphate dihydrate, Ion exchange resin and so on.[43]

The remineralizing efficacy of nano-hydroxyapatite has derived special attention in recent years. Nano-hydroxyapatite has been accepted as one of the most bioactive and biocompatible materials.[40,42,44] These particles are similar to the apatite crystals of enamel in morphology and crystal structure.[18,27,32,42,44,50] It's mode of action is due to the adsorption of hydroxyapatite particles on the

tooth surface. The adsorbed particles inhibit enamel dissolution acting as a sacrificial layer or buffering agent, being more readily dissolved than the underlying enamel.

Hydroxyapatite layer can also elevate the calcium and phosphate source at the enamel surface, reducing the degree of under saturation during the acid challenge. Thus it reduces the dissolution of enamel[12].In contrast to fluoride, nano-hydroxyapatite can influence the tooth enamel remineralization at both the nanocrystalline and ionic levels.[15]

In the present study design, 20nm size (mK.Nano) nano-hydroxyapatite particles were chosen to evaluate its remineralizing effect on early enamel lesions and compare it with that of sodium fluoride. The objective of the present study was to determine the effects of nano-hydroxyapatite concentration on the early enamel lesions over a range of time periods under dynamic pH cycling conditions. This remineralization study has allowed demineralization and remineralization to occur in a dynamic equilibrium. As per literature arrested lesions will exhibit increased surface hardness and missing porosity.[29] In this study analysis of

surface microhardness and surface roughness have been done for the assessment of remineralization.

In a study by Haung et al, various concentrations such as 1, 5, 10 and 15%wtof nano-hydroxyapatite were investigated, and it was concluded that no difference existed between the 10%wt and 15%wt concentrations. An identical result was observed in the study done by Xiangcai et al. Based on these findings, 10%wt concentration of nano-hydroxyapatite was taken in to consideration in this study. Anut Itthagarun et al in his study confirmed that toothpaste containing 10%wt nano-hydroxyapatite remineralized artificial enamel caries lesion.[15] Therefore an intermediate concentration of 8%wt nano-hydroxyapatite was also included in this study along with 5%wt and 10%wt to evaluate the optimum concentration of nano-hydroxyapatite.

The inclusion criteria used were freshly extracted central incisors devoid of cracks, caries and hypoplasia. Each 4×4 mm enamel core had sufficient space for multiple Vickers diamond-shaped indents onto the flat surface. Using this technique, the specimen surfaces were impressed with a diamond indenter at a certain load for a certain period of time.[3] Here 200gf load and 15s

dwell time have been used to confirm the presence of sound enamel in the polished sample.[20]

After load removal, diagonals of the indentation were measured with an optical microscope and the hardness number recorded. This is the ratio between the indentation load and the area of the residual impression, which depended on the indenter shape. The minimum spacing of indents was 2.5 times the indent diagonal. Each test done with the same load and time was conducted three times.[3] An average of three readings for each test condition was recorded as the baseline value which was between 270-320 VHN (similar to that of sound enamel)[24] which is in accordance with previous studies by Maria del pilar et al.

According to R. L. Karlinsey et al, the Vickers indenter penetrated into the white-spot lesion about twice as much as knoop indenter**.** Thus, surface microhardness was assessed using the Vickers indenter in order to facilitate further cross-sectional microhardness analysis, without extensive cracking.[20]

The enamel blocks after recording the baseline value were immersed in demineralizing solution and kept in an incubator at 37°c

to create artificial carious lesions. The time of 72 hours was chosen to induce artificial caries-like lesion on the enamel. The enamel specimens showed measurable caries-like subsurface lesions without surface erosion after 72 hours (IMAGE2).This allowed the evaluation of mineral loss or gain by determining surface microhardness. The demineralizing solution contained an acidic buffer which was composed of 2.2mM Calcium nitrate$(CaNO_3)_2$, 2.2 mM potassium dihydrogen ortho phosphate (KH_2PO_4), and 0.1 ppm sodium fluoride (NaF) and 50mM of acetic acid at pH of 4.5.[2,5,13] The Vickers hardness number was again recorded to ensure the reduction in microhardness which ranged from 20-40VHN.

The pH-cycling experiment involved exposure of enamel samples to treatment, remineralizing and demineralizing solutions to simulate the PH changes in the oral environment. This offered high level of scientific control, lower variability and the smaller sample size required.[2] The pH cycling model followed in this study was similar to that reported by white.[46] In this model, the dynamic cycles of de- and remineralization are simulated by sequentially immersing enamel specimens in acidic (demineralizing) and supersaturated (remineralizing) buffer solutions (TABLE 1).

The enamel blocks were subjected to 2hours of demineralization on each day of pH cycling. This was because frequent episodes of long duration lowered pH, will be responsible for demineralization in the clinical scenario. In a review reporting the different pH cycling studies, they have concluded that when the focus is more on remineralization, the samples were kept in the demineralizing solution for a lesser time. Our study design corroborated as reported by Buzalaf et al[2] in the review. Hence, these solutions were prepared with a higher pH and lower concentration of acid and ions. Whereas, the specimens were kept in the remineralizing solution for longer duration, with the ionic concentration and the pH remaining the same.

The remineralizing solutions used in the study were made to replicate supersaturation by apatite minerals found in saliva (Ten Cate and Duijster). The remineralization solution contained calcium and phosphate at a known degree of saturation 1.5 mM Calcium chloride ($CaCl_2$) and 0.9 mM potassium dihydrogen orthophosphate (KH_2PO_4) to replicate super saturation combinations of demineralization and remineralization by apatite minerals found in saliva.130mM Potassium chloride (KCl) was used to provide background ionic

strength and 20mM HEPES ***(4-(2-hydroxyethyl)-1-piperazine-ethane-sulfonic acid)*** was used as a buffering agent at pH of 7.0.[2]

Nano-hydroxyapatite powder in the particle size of 20 nm was purchased from mk Nano impexcorp, Canada. Treatment solutions were made as 5%wt, 8%wt and 10%wt nano-hydroxyapatite suspension liquid in distilled water, with pH adjusted to 7 using 2M HCL similar to the study done by Huang et al. The positive control was 1000ppm sodium fluoride aqueous and the negative control was distilled water. The pH-cycling was done for 12 days and temperature maintained at 37°c in an incubator. The demineralizing and remineralizing solutions were freshly made every third day where as treatment solutions were made daily. This was done to simulate the daily acid challenges in the oral cavity.[13]

Microhardness measurements were carried out at the end of third day, sixth day, ninth day, and twelvth day. This was done to ensure, whether the longer exposure time can produce remineralization, resulting in stronger and greater acid-resistant enamel.[16] The response variables measured the mineral gain in the

lesions as a result of the treatment with the different concentrations of nano-hydroxyapatite with sodium fluoride as the positive control.

During the 12-day cycling period, increased porosity and solubility of the enamel would occur due to repeated exposures to the acid challenge. The extent of remineralization from exposure to the distilled water would be limited due to the relatively poor mineral nucleation ability of calcium and phosphate of the remineralizing solution relative to other groups. Robinson et al have substantiated that, when there is no effective nucleation, the development of acid-resistant mineral does not result, leading to leaching of apatite constituents such as calcium, phosphate, and carbonate from the enamel tissue.[20]

Surface microhardness values by micro indentation confirmed the ability of nano-hydroxyapatite for remineralization. This was evident at each time point in the pH-cycling as different concentrations were directly linked to the distinct effects on remineralization. When the size of any particle decreases, it's surface area and atomicity proportion increases. Kaehler et al has contributed this property to the bioactive and biocompatible properties of nano-hydroxyapatite. This can be taken in to account in this study as

these factors increased the potential of nano-hydroxyapatite to directly fill up the micropores on demineralised teeth.[13]

As early enamel lesions are more porous than sound enamel, it allowed for a greater penetration of these particles.[47] The nano-hydroxyapatite filled up the enamel defects and acted as a template in the process of precipitation. This would have attracted a large amount of calcium and phosphate from the remineralizing solution to bridge for the vacant spaces of enamel crystals there by facilititating crystal integrity and growth.(in accordance with Huang et al). In the present study, this was observed in three-dimensional atomic force microscopic image of remineralization of artificial enamel lesions. (IMAGE-4,5,6)

Percentage of surface microhardness recovery was highest for Sodium fluoride followed by 10%, 8% and 5%wt nano-hydroxyapatite ($P<0.001$) -(TABLE-2). In all the treatment groups when compared from baseline to various time intervals, there was statistically significant increase in the microhardness values. ($p<0.001$)-(TABLE-3,4,5,6,7).

Concerning nano-hydroxyapatite, as the concentration increased, rate and amount of nano-hydroxyapatite precipitation

increased along with the calcium and phosphate deposition.[13] kim et al also found an increase in surface hardness of the demineralized enamel after subjecting the samples to an increasing nano-hydroxyapatite concentration after adding to nano-hydroxyapatite mouthwash. similarly in the present study, it was revealed that, for all treatment groups, microhardness recovery percentage increased remarkably in first 6 days of pH cycling as depicted in the GRAPH-1.

After the first 6 days of pH cycling, as a result of remineralization of the outer enamel region, the deposition of nano-hydroxyapatite on the surface layer would have blocked the surface pores. This could restrict diffusion into the lesion over the short term of remineralization. But, this deposition would have come to a stable level even though pH cycling model continued for 6 more days.[13]

Huang et al in his study have also reported that there is no significant difference in remineralization after 6 days of pH-cycling. Tencate et al have reported that lesions can be rehardened by deposition of hydroxyapatite that is initially deposited near the surface layer, which is then gradually transferred inward and finally precipitated in the dark zone in the long-term remineralization.[39]

Atomic force microscopic evaluation was performed for the analysis of surface roughness in this study. Wennerberg et al stated that though scanning electron microscopy is routinely used, it is not a quantitative method and is prone to subjective interpretation. M.Farina et al concluded in his study that atomic force microscopy gives high contrast, high resolution images and it is as a tool for complementary or new structural information.[7]

Marshall et al demonstrated that Atomic force microscopy as a powerful tool for observing demineralization, drying, bonding processes and mechanical properties of calcified tissues. Lippert et al in his study observed the demineralization and remineralization of surface softened enamel with high accuracy. Robinson et al reported that, high –resolution atomic force microscopic images of sound human enamel revealed spherical domains within crystals, arranged as layers of hexagons or as a shallow spiral on the sound enamel surface.[49] Hence in this present study also, surface analysis of demineralized and remineralized enamel was done using atomic force microscopy.

The characterisation of mineralised tissue is one of the most active areas of Atomic force microscopy in biomaterials science. The

unique resolution of this microscope depends on the ultra sharp probe. This probe consists of a microfabricated pyramidal Silicon Nitride tip with radii of typically 4-60nm.This tip is fixed at the end of a flexible cantilever and accurate ceramic piezoelements. This allows the sample scanning in the x-y plane at the level of sub-nanometric precision. The cantilever deflects in the z-direction according to the surface topography during tip scanning over the sample surface. A four segment photodiode detects the cantilever deflection through a laser beam. The laser beam is focused on and reflected from the rear of the cantilever. From each point of the surface, electrical signal is processed by a computer .The piezoscanner utilises this feedback signal to maintain a constant force on the tip and this information is transferred in to a topographic surface image.[17] Depending on the interaction signs between the tip and the sample, it works in two different modes: Contact (repulsive) and non contact (attractive) modes.[21]

Non contact mode was chosen in this study as it could produce image by offering the lowest possible interaction between sample and tip. It was because forces involved are much lower in the non contact mode (10^{-12} compared with 10^{-9} N). In non contact mode, cantilever

tip is placed at the attractive force region and detection of force gradients is done. The force gradients are detected either from the resonance frequency shifts of the cantilever or the amplitude and the phase of the cantilever.[21]

The scanning rate chosen was 0.1 Hz and 256 lines per sample. The surface roughness values were provided with ***XLE*** systemic software and the parameters were recorded. Two roughness parameters recorded were **Rrms and Rz.** The root mean square roughness (Rrms) represents the height distribution relative to the mean line. Rz is the average maximum peak -to-valley height of five consecutive sampling depths. (Eliades et al).[49]

The roughness parameters Rrms and Rz were recorded in this study to characterise the nano-scale mechanical properties of surface deposits. To gain information on deep or shallow grooves and on the profile of the irregularity as peaks or valleys, Rrms and Rz values were taken in to consideration. The Rrms and Rz values decreased appreciably and were statistically significant after remineralization for all the treatment groups except distilled water group (Negative control)-(TABLE 8&9). Similarly, percentage difference of Rrms and Rz values showed appreciable decrease in surface roughness for all

the treatment groups with the exception of negative control (GRAPH 2&3).

In the sodium fluoride group (positive control) atomic force microscopic 3D-image revealed raised mountain like structures and decreased surface roughness. This can be attributed to the deposits of calcium fluoride or fluorapatite crystals as discussed before[33] (IMAGE-3).

In all the groups of nano-hydroxyapatite, similar morphological changes and decreased surface roughness were evident with maximum changes with 10% wt nano-hydroxyapatite followed by 8% and 5%wt nano-hydroxyapatite particles respectively (IMAGE-4,5,6).This study has confirmed the ability of nano-hydroxyapatite particles to remineralize early enamel lesions by demonstrating increase in surface hardness and decrease in surface roughness.

Regarding the future prospects of this material, Peter Tschoppe et al[40] (2011) have proved higher remineralizing effect of toothpastes containing nano-hydroxyapatite when compared to amine fluoride toothpastes.[49] N.Roveri et al in his study concluded that synthetic

biomimetic carbonate-Hydroxyapatite nanocrystals can favour remineralization.[34] In studies done by Kim et al.(2007) nano-hydroxyapatite was used in mouthwash, toothpaste and chewing gum (Kim et al, 2007; Jeong et al, 2006; Kjolhede and Gyldenvang, 2009) respectively. The above studies confirmed the ability of nano-hydroxyapatite for remineralization. It was concluded that all these materials had the potential to remineralize enamel, although additional substances were present in the those products that could have a synergistic effect with nano–hydroxyapatite. This observation coincides with the similar study of Haung and Xiangcai, in which fluoride was added to nano-hydroxyapatite.[10]

SUMMARY

Dental caries can be regarded as a 'pandemic' disease owing to its global prevalence. From the surgical approach for the elimination of carious lesion, it has been now shifted to the development of methodologies, for the non- invasive treatment of these lesions. This study determined the effect of nano-hydroxyapatite concentrations on early enamel lesions under dynamic pH-cycling conditions. As increase in surface hardness and decrease in surface roughness are evidences for remineralization, both these criterias were taken in to account in this study.

Early artificial caries like lesions were prepared in human enamel with an acidic buffer. Sodium fluoride (positive control), distilled water (negative control) and three different concentrations of nano-hydroxyapatite (5%, 8% and 10%wt) were selected as the treatment agents. The pH cycling model was used in this study to simulate the oral environment's acid challenges. All the groups were subjected to various treatment solutions as per the pH cycling model. Surface microhardness (SMH) measurements were done before/after demineralization and after 3, 6, 9 and 12 days of application, and the percentage surface microhardness recovery (%SMHR) was

calculated. The % SMHR in nano-hydroxyapatite groups was significantly greater than that of negative control but lesser than sodium fluoride. The surface microhardness recovery decreased with decreasing concentrations of nano-hydroxyapatite. Atomic force microscopy was done for the samples before and after remineralization. Surface roughness parameters clearly demonstrated decrease in surface roughness, the highest being with 10%wt nano-hydroxyapatite among the nano-hydroxyapatite groups.

The result of the present study were subjected to statistical analysis to interpret the surface hardness and surface roughness in each group and also between the groups. ONE-WAY ANOVA and POST HOC TUKEY tests were used for statistical analysis in the present study.

It was concluded that nano-hydroxyapatite has the potential to remineralize initial artificial caries like lesions in the enamel. A concentration of 10%wt nano-hydroxyapatite can be regarded as an optimum concentration for remineralization of early enamel caries.

CONCLUSION

Within the limitations of this invitro study on the remineralization of artificial enamel lesions, it can be concluded that:

1. The surface hardness of artificially created enamel lesions increased after remineralization using nano-hydroxyapatite at various concentrations and at various time periods.
2. The atomic force microscopy demonstrated significant decrease in surface roughness of remineralized artificially created enamel lesions in the following ranking: **Sodium fluoride>10%wt NHA>8%wt NHA>5%wt NHA>Distilled water**.
3. Among the various concentrations of nano-hydroxyapatite tested, 10%wt proved to be an optimum concentration followed by 8%wt and 5%wt.
4. An increase in surface hardness and decrease in surface roughness on remineralized early enamel lesions were observed in this study, even though values were lesser for nano-hydroxyapatite when compared to sodium fluoride.

5. This study can be considered as one among the very few studies that have evaluated the remineralization using **Atomic force microscopy**.

The ongoing researchers are evolving a paradigm shift in caries management which has opened up newer vistas, to cure this multifactorial disease by microinvasive technology. Thus the conservative surgical management of early enamel lesions is getting transformed to non invasive medical approach.

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